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The goal of this research is to understand the regulation of Ras-mediated signaling in *C. elegans* vulval development. We describe the genetic and molecular characterization of two genes, *sur-6* and *sur-8*, which both positively regulate *ras*-mediated signaling.

sur-6 (*su*ppressor of *r*as) was identified as a suppressor of the Multivulva phenotype caused by an activated *let-60 ras* mutation. Genetic studies indicate that *sur-6* is a non-essential, positively acting regulator of *ras*-mediated signaling and it acts in a dosage-dependent manner. *sur-6* likely acts downstream of or in parallel to *let-60 ras* and upstream of *lin-45 raf* together with the *ksr-1* at a branch point at the level of *ras* or *raf*. We have cloned *sur-6* and show that it encodes a highly conserved regulatory B subunit of the serine/threonine protein phosphatase 2A.

sur-8 is defined by two loss-of-function alleles and genetic characterization indicates that, like *sur-6*, it plays a non-essential but activating role in *ras* signaling downstream of or in parallel to *let-60 ras* and upstream of *lin-45 raf*.

However, unlike *sur-6*, *sur-8* most likely acts in a branch of the pathway distinct from *sur-6* to regulate *ras* signaling activity. *sur-8* has been cloned and is predicted to encode a novel protein.

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"Genetic and Molecular Analysis of Suppressors of Ras Mutations"

INTRODUCTION:

The Ras family of proto-oncogenes encodes a class of small GTP-binding proteins that play a central role in signal transduction cascades controlling cell proliferation and differentiation during development. Ras proteins act as molecular switches by cycling between inactive, GDP-bound states and active, GTP-bound states. Ras proteins are activated by guanine nucleotide exchange factors (GNEFs), which accelerate the replacement of bound GDP with GTP, and are deactivated by GTPase activating proteins (rasGAPs), which promote GTP hydrolysis by Ras. Ligand activation of receptor tyrosine kinases (RTKs) leads to increased levels of activated Ras. One of the biochemical functions of activated Ras is to recruit the protein serine/threonine kinase Raf to the cell membrane where Raf becomes activated. Once activated, Raf phosphorylates and activates MAPK/ERK kinase, which then phosphorylates and activates MAP kinase, which modifies the activity of various proteins including transcription factors. Modification of MAP kinase targets can cause changes in the expression of genes that direct cell proliferation and differentiation.

Although the mechanisms are understood by which the main components of the Ras pathway transduce a signal from the membrane to the nucleus, little is known about positive and negative regulation of the pathway. For example, the mechanism of Raf activation is poorly understood. Studies in mammalian cells suggest that Raf may need to be activated by phosphorylation once it is recruited to the membrane by Ras. Furthermore, the mechanism of the down-regulation of the Raf, MEK, MAP kinase cascade downstream of Ras is unknown. Phosphatases that specifically down-regulate all or one of these kinases upon kinase activation have not yet been identified.

There is mounting evidence that the ras pathway interacts with other signaling pathways as a way to integrate information from multiple signals for the proper cellular response. Each component of the ras pathway may define a branch point where signals, both positive and negative, from other signaling cascades converge with the ras pathway or where the ras pathway feeds into other signaling pathways in the cell. This idea is supported by recent mammalian studies showing that Ras may define a branch where the Ras pathway feeds into other signaling pathways: The Ras effector domain may directly target PI-3 kinase, rasGAP, and neurofibromin in addition to Raf, although the significance of these interactions remains poorly defined. In addition, Raf may integrate signals not only from Ras, but from protein kinase C and c-mos as well, and may thus define a point where multiple signal transduction pathways converge. Further downstream, MAPK may define a point where two signaling pathways converge and diverge again; JAK of the JAK/ STAT signaling pathway may activate MAP kinase, and MAP kinase may co-activate STAT. Although these putative branches have been identified in tissue culture systems, these components have yet to be shown to regulate ras-mediated signaling during cell fate specification in vivo.

Dissecting the Ras pathway in genetic model systems has provided a powerful means to identify and characterize genes that act in this pathway. In *C. elegans*, a ras-mediated signal transduction pathway controls the development of the hermaphrodite vulva. The vulva is an opening in the ventral uterus used for egg laying and copulation. During development, of the six vulval precursor cells (VPCs) the three closest to the neighboring gonad adopt vulval cell fates and they undergo three rounds of division followed by morphogenesis to form the vulval structure. The remaining three cells adopt non-vulval cell fates and instead divide only once and then fuse with the surrounding hypodermis. The VPCs are initially equivalent in developmental potential and adopt vulval cell fates only when induced by a signal from the gonad. VPCs that are far from the gonad receive no inductive signal and they adopt the hypodermal fate.

The gonad signal is an EGF-like protein encoded by *lin-3*, which activates a RTK on the surface of the VPCs encoded by *let-23*. *let-23* activates Ras, encoded by *let-60* through the SH2-SH3 containing adapter protein, encoded by *sem-5*. *let-60 ras* activates a kinase cascade encoded by *lin-45* raf, *mek-2* MEK, and *sur-1/mpk-1* MAP kinase. *sur-1/mpk-1* MAP kinase modifies the activities of proteins including the transcription factors *lin-1* and *lin-31*, resulting in changes of gene expression and ultimately the adoption of vulval cell fates. See figure.

Mutations that result in the mis-specification of vulval cell fates have defined many genes necessary for normal vulval differentiation. Loss-of-function (lf) mutations in any of the positively acting components of this pathway result in reduction or loss of vulval induction. In this case, fewer than three VPCs adopt vulval cell fates leading to a Vulvaless (Vul) phenotype. Loss-of-function mutations in negatively acting components lead to an increase of vulval induction, resulting in a Multivulva (Muv) phenotype caused by the formation of pseudovulvae. A gain-of-function mutation in *let-60 ras* (*n1046gf*) constitutively activates the ras pathway in all six VPCs, resulting in a Muv phenotype in 85% of animals homozygous for the mutation. The *n1046* allele encodes a Ras protein in which glutamic acid is substituted for glycine at position 13 in the GTP binding loop. This mutant Ras has increased activity presumably because of reduced GTPase activity. The Muv phenotype caused by the activated ras mutation can be suppressed by mutations in components acting downstream of ras which reduce ras-mediated signaling in all six VPCs. With the main genes in the pathway identified and their mechanisms of action understood by inference from biochemical studies of their mammalian homologues, the next goal is to understand how each component is regulated during vulval signaling.

To identify genes that positively and negatively affect ras-mediated signaling I have screened for mutations in genes that suppress the Muv phenotype of an activated ras mutation. Mutations that suppress the Muv phenotype are expected to cause a reduction in signaling through the ras pathway resulting in a decrease in VPC induction. Using this screen we expect to identify mutations in genes that either regulate components downstream of Ras, or regulate the expression of Ras or act in parallel pathway during signaling. For example, loss-of-function mutations in positively acting genes or gain-of-function mutations in negatively acting genes should suppress the Muv phenotype. This screen has already been successfully used to identify several downstream components of *ras* signaling including *lin-45* Raf, *mek-2* MEK, *sur-1/mpk-1* MAP kinase and *sur-2* (novel). In addition, a non-essential, positively regulating novel protein serine/threonine kinase encoded by *ksr-1* was identified in this screen. Because this screen is not yet saturated, it is likely that that this screen will continue to be a powerful tool in the identification of novel genes that regulate components downstream of Ras. In addition regulators of ras signaling may be identified only in a suppressor screen and not a

traditional screen since mutations in these genes may not cause any apparent phenotypes on their own.

BODY:

Results and Discussion

Genetic and Molecular Analysis of *sur-6*

Genetic characterization *sur-6* indicates that it is a positive modulator of the *ras* pathway. *sur-6* is defined by a single allele, *ku123*, isolated as a dominant suppressor of the Multivulva phenotype caused by *let-60(n1046gf)*. *ku123* suppresses the Multivulva phenotype to wild-type and causes no vulval phenotype in an otherwise wild type background. Genetic dosage studies indicate that *ku123* is a strong loss-of-function allele since *ku123* homozygotes suppress the Multivulva caused by the activated *ras* almost as well as animals with *ku123* in trans to a deficiency of the locus. The *sur-6* locus is haploinsufficient: animals with *sur-6 (+)* in trans to a deficiency of the locus display a suppressed phenotype, indicating that one wild type copy is not sufficient for efficient *ras*-mediated signaling (see below). Thus, *sur-6* plays a non-essential but activating role in *ras*-mediated signaling and signaling is sensitive to *sur-6* dosage.

genotype	Multivulva %
wild type	0%
let-60(n1046)	88%
sur-6 (ku123); let-60(n1046)	6%
sur-6 (ku123)/ +; let-60(n1046)	10%
sur-6 (ku123)	0%

Epistasis analysis indicates that *sur-6* acts between, or more likely, in parallel to *let-60 ras* and *lin-45 raf*. *ku123* can suppress the Multivulva phenotype caused *by let-60(n1046gf)* and can also suppress the Multivulva phenotype of a loss-of-function *lin-15* mutant. Since *lin-15* acts upstream of *ras* at the level of *let-23* RTK, this result is not only consistent with a role for *sur-6* downstream of *ras*, but indicates that *ku123* is not an allele specific suppressor; it can suppress *ras* pathway signaling when *ras* is wild type. *Ku123* does not suppress the Multivulva phenotype caused by an activated *raf* transgene or a loss-of-function mutation in the putative downstream transcription factor *lin-1*. Thus, *sur-6* acts upstream of *raf* and downstream or in parallel to *ras*.

genotype	Multivulva %
let-60(n1046)	88%
sur-6(ku123); let-60(n1046)	6%
raf (gf)	43%
sur-6(ku123); raf (gf)	47%
lin-15(n765)	95%
sur-6(ku123); lin-15(n765)	6%

ku123 enhances the Vulvaless and Lethal phenotypes caused by weak loss-of-function mutations in the *ras* pathway components *lin-45 raf* and *sur-1/mpk-1* MAP kinase. In double mutants vulval induction (the percent of VPCs induced relative to wild

type) and viability are reduced, but not completely eliminated, suggesting that either *ku123* is not a null allele and is still partially functional or that *sur-6* normally only stimulates the ras pathway moderately.

ku123 does not synergyse with a strong loss-of-function mutation in *ksr-1*, a positive modulator of the *ras* pathway that also acts at the level of *ras* or *raf*. Double mutants and single mutants are display wild type vulval induction. These results suggest that *sur-6* may act together with *ksr-1* to activate the pathway at the level of *ras* or *raf*.

genotype	vulval induction%	Lethal%
sur-6 (ku123)	100%	0%
sur-1(ku1)	98%	7%
ksr-1(ku68)	100%	24%
sur-6 (ku123); sur-1(ku1)	66%	74%
sur-6 (ku123); ksr-1(ku68)	100%	17%

I have cloned *sur-6* using a positional cloning strategy. First, I used genetic mapping to define a small region for *sur-6* on linkage group I that is represented by DNA from overlapping cosmids and YACs. I tested twelve cosmids in the region for their ability to revert the suppressed phenotype of *ku123*; *let-60(n1046gf)* animals to the Multivulva phenotype using DNA-mediated germline transformation. One cosmid, KO2A11 rescues *ku123* mutants. KO2A11 contains a 40kb insert that has been sequenced by the C. elegans genome sequencing project. I identified a 6kb rescuing subclone from cosmid KO2A11, which contains a single predicted gene (see appendix). To identify sur-6 cDNA, I probed a mixed stage C. elegans library using a probe derived from the rescuing DNA subfragment. From one million cloned screened, I identified 16 positive clones and determined the DNA sequence of the two longest clones. Each clone contains a single predicted open reading frame. To prove that this cDNA corresponds to *sur-6,* I sequenced the gene coded for by this cDNA from genomic DNA derived from *ku123* mutants and identified a single molecular lesion in an exon.

sur-6 is predicted to encode the regulatory subunit B of protein phosphatase 2B (PP2B). PP2B is a highly conserved heterotrimeric serine/threonine protein phosphatase made up of a heterodimer catalytic core and one of several associated regulatory subunits. PP2A involved in a variety of cellular and developmental events ranging from cell fate specification in Drosophila bristle cells to regulation of anaphase progression and bud site selection in yeast. Regulatory subunits are thought to control the activity, or localization of the catalytic core. Because sur-6 is a positive regulator of ras signaling, we believe that it may have one of two roles: It either acts as a negative regulator of a negatively acting catalytic core or as a positive regulator of a positively acting catalytic core. We are currently testing these models by determining the effects of overexpression of *sur-6* on ras pathway signaling.

Suppressor Screen of Multicopy Activated *ras*

In order to find new alleles of *sur-6* and to define other genes that regulate the *ras* pathway downstream of *ras*, I have screened for additional suppressors of the Multivulva phenotype caused by the *let-60(n1046gf)* mutation. To increase the efficiency of this screen I have used a strain that expresses an integrated *ras(n1046gf)* transgene. Unlike *let-60(n1046gf)* homozygotes, which display a 88% penetrant Multivulva phenotype, the transgenic animals display a 100% penetrant Multivulva phenotype such that every animal in Multivulva. As a result, fewer fake suppressed animals are picked during a suppressor

screen so more genomes can be screened. In addition, both dominant and recessive suppressors can be identified easily. In an ethyl methanesulfonate (EMS) screen of 22,000 haploid genomes, I identified 11 mutations representing at least four genes each suppressing the Multivulva phenotype to less than 5%. I identified three alleles each of *mek-2* MEK and *lin-45 raf* indicating that this new screen was suitable for identifying *ras* pathway components. In addition, four other alleles mapped on the left side of IV and their gene identity has not been determined. The remaining allele, *ku167*, was mapped to a genetic map position on chromosome IV where no known genes involved in the Ras pathway were located. This allele defines the sur-8 gene.

Genetic Analysis of *sur-8*

sur-8 which is defined by a single allele, *ku167* was isolated as a recessive suppressor of the Multivulva phenotype caused by a transgene expressing the activated *ras* allele *let-60(n1046gf)*. In animals homozygous for the *ku167* allele vulval induction is completely wild type, indicating that if *ku167* is a loss-of-function allele, *sur-8*, like *sur-6,* may play a non-essential but activating role in *ras* signaling. To determine the genetic nature of the *ku167* allele, we compared the phenotype of *ku167* homozygotes with animals with *ku167* in trans to a deficiency that uncovers the locus in a sensitized genetic background. The Vulvaless phenotype in a *ku167 ksr-1(ku68)* double mutant is slightly enhanced when *ku167* is in trans to a deficiency of the locus, indicating that *ku167* is a loss-of function allele, but probably not a null allele. *ku167* homozygotes display no vulval phenotype or other obvious phenotypes in an otherwise wild type background. Together, this data suggests that the *sur-8* is a non-essential but positively regulating gene acting in the *ras* pathway during vulval development.

genotype	Multivulva %
wild type	0%
let-60(n1046)	88%
sur-8(ku167) let-60(n1046)	4%
sur-8(ku167)	0%

In order to determine the relationship between *sur-8* and other genes acting in the ras pathway, we have conducted epistatic analysis with mutations that cause a Multivulva phenotype. Epistatic analysis indicates that *sur-8* acts downstream or in parallel to *let-60 ras* and upstream of the *lin-45 raf.* like *ku123*, *ku167* can suppress the Multivulva phenotype caused *by let-60(n1046gf)* and can also suppress the Multivulva phenotype of a loss-of-function *lin-15*, indicating that *ku167* is not an allele specific suppressor; it can suppress *ras* pathway signaling when *ras* is wild type. *ku167* does not suppress the Multivulva phenotype caused by a loss-of-function mutation of *lin-1* or an activated *raf* transgene. Because *ras* and *raf* have been shown to directly interact, I favor a model where *sur-8* defines a branch point either feeding into *raf* or out of *ras*.

genotype	Multivulva %
let-60(n1046)	88%
sur-8(ku167) let-60(n1046)	4%
raf (gf)	43%
sur-8(ku167); raf (gf)	50%
lin-15(n765)	95%

sur-8(ku167); lin-15(n765) 49%

ku167 strongly enhances the Vulvaless and Lethal phenotypes caused by a weak loss-of-function mutation in *sur-1/mpk-1* MAP kinase or a strong loss-of-function mutation in *ksr-1*. Single mutants display wild type vulval induction and almost no lethality while double mutants (from heterozygous mothers) are completely Vulvaless and all of their progeny die as larvae. These results contrast with those for *sur-6(ku123)*, which synergises moderately with *sur-1/mpk-1* MAP kinase but not with *ksr-1*. Thus, while *sur-6* likely acts together with *ksr-1* to positively influence *ras* signaling, *sur-8* likely defines a distinct branch point.

genotype	vulval induction%	Lethal%
sur-8(ku167)	100%	0%
sur-1(ku1)	98%	7%
ksr-1(ku68)	100%	24%
sur-1(ku1); sur-8(ku167)	0%	100%
sur-8(ku167); ksr-1(ku68)	4%	85%

I cloned *sur-8* using standard positional cloning techniques. *sur-8* maps to a small genetic interval on the left arm of chromosome IV. A single cosmid, which maps to that genetic interval, was shown to carry the *sur-8* gene because it rescued the mutant phenotype caused by the *ku167* allele when expressed in *C. elegans* (see appendix). The full length *sur-8* cDNA was identified by probing a mixed stage *C. elegans* cDNA library with a 2 kb fragment from a rescuing cosmid subclone. Of 2 million library clones screened, 10 *sur-8* clones were identified. The two longest clones (2.1 kb) were sequenced and contain a single open reading frame, which is predicted to encode a novel protein of 559 amino acids. Point mutations in this open reading frame are found in both *sur-8* alleles, confirming that these clones represent *sur-8* cDNA. The predicted protein encoded by *sur-8* is contains 18 leucine rich repeats (LRR). The LRRs form two clusters separated by three repeats with a non LRR consensus. LRRs are found in a variety of proteins with diverse biological functions and are thought to mediate protein-protein interactions. The LRRs of SUR-8 most closely resemble those of yeast adenylate cyclase.

We have isolated the *sur-8* homologue in humans and mice. Expressed sequence tags (EST) with a high degree of homology to the C-terminus of SUR-8 were identified from a Genbank search. Oligonucleotides corresponding to these ESTs were used to amplify, using 5' RACE (rapid amplification of cDNA ends), the remaining 5' end from a human brain library and a mouse liver library. The human and mouse homologue sequences share 60% identity at the amino acid level.

C. elegans SUR-8 specifically binds LET-60 *ras* in the yeast two hybrid system. SUR-8 fusion proteins bind to both LET-60 *ras* and the gain of function mutant, LET-60 (G13E), but fail to bind to other *ras* pathway components tested, LIN-45, KSR-1, MPK-1, or MEK-2. We are currently testing whether the human homologue binds to any of the human RAS proteins identified thus far.

Methods and Procedures:

Construction of a *let-60 ras (gf)* transgenic strain for screening suppressors of Ras:

To construct the strain with completely penetrant Multivulva phenotype, we transformed wild type *C. elegans* with cloned *let-60 ras(n1046)* mutant genomic DNA fragment. Transformation was performed by microinjecting DNA to the gonad of C. elegans adult. The host strain was of genotype *dpy-20*. The *dpy-20* mutation causes the worms to have a Dumpy phenotype. The injection solution contained three plasmids: pMH132 (1ug/ml) which contains the activated *let-60 ras (n1046)* mutant gene derived from pMH106, pMH86 (10ug/ml) which contains the dpy-20(+) genomic DNA fragment, and bluescript SK(+) (100ug/ml) serves as a carrier. The dpy-20(+) plasmid serves as a transformation marker that will rescue the Dumpy phenotype of the host strain.

To obtain stable lines carrying an extra array of injected DNA, non-Dumpy but Multivulva progeny from injected animals were selected in the F1 generation three days later. Non-Dumpy Multivulva animals were selected again in the F2 generation three days later. The F2 transformants stabily carried the injected DNA as an extrachromosomal array .

To integrate the array into the genome, transgenic animals from such lines were gamma-irradiated at a dose of 3600 rads, then their F1 non-Dpy progeny were picked singly to plates to identify those in which the trans gene had integrated into the genome (giving 75% transmittance to the F2). Lines bearing integrated transgenes were obtained at a frequency of approximately 1 in 200 F1s picked. Two transgenic lines, *kuIs13* and *kuIs14* [*let-60(n1046)*] were obtained. Each line was backcrossed one time to the parent *dpy-20* strain to remove any other background mutations before analysis.

Screen for suppressors of the Multivulva phenotype of kuIS14:

let-60(kuIs14) homozygotes are nearly 100% Multivulva and were used as a parental atrain to screen for suppressors of the Multivulva phenotype. Several plates of mixed staged kuIs14 animals were collected in a 15ml tube. After washed once in a salt solution (M9), they were treated with mutagen EMS at 50mM (20 ul concentrated EMS added to 4 ml worm solutions). The tube was gently shaken in a hood for four hours. The worms were then washed three times with about 10ml M9 solution to wash off EMS. The worms were placed on a new culture plate. Two-to-three hours later, fourth larval stage worms were picked and individually placed on culture plates. For each screen we use about 200 plates to screen the progeny of 200 treated worms. These plates were incubated in a 20°C incubator

Three days later, we estimate how many F1 progeny a single worm produces on average. About 7-8 days later, we screen for suppressed F2 animals (non-Mutivulva) from each plate under a dissecting microscope. Non-Multivulva animals were selected and individually placed on a new plate labeled with a number. Candidates from the same parent place may not be independent mutants and thus assigned the same plate number. If the picked candidate continued to produce mainly non-Multivulva progeny, this line of worms considered a good candidate to contain a suppressor mutation.

Outcrossing Candidates:

Candidate suppressors were outcrossed to remove any additional mutations from the genome. Prior to outcrossing, each candidate had the following genotype: *dpy-20/dpy-20; let-60(kuIs14)/let-60(kuIs14)*; suppressor/suppressor. These animals were mated with males with a genotype of *let-60(n1046 gf) +/+ dpy-20(e1282); him-5(e1490)/him-5(e1490)*. The *dpy-20* gene is extremely close to the *let-60* gene and thus a good balancer in the strain. The *him-5* mutation leads to male production. F1 progeny having a genotype of

let-60(n1046 gf) +/+ dpy-20; let-60(kuIs14)/+; suppressor/+ were selected. Since most of the suppressors are recessive or semidominant, these animals were Multivulva. F2 non-Multivulva progeny were selected to obtain strains of *let-60(n1046)/let-60(n1046); suppressor/suppressor*. These strains continued to produce mostly non-Multivulva animals. Further outcrosses were done in similar way using the same male strain. Since the male strain has the *him-5* mutation, we also obtain strains of genotype *let-60(n1046)/let-60(n1046); him-5(e1490)/him-5(e1490)* to facilitate mapping. In total, 11 candidates were outcrossed at least four times and each one supressed the Mutivulva phenotype to less than 5%.

Genetic Mapping:

Two point genetic mapping was used to assign each suppressor to one of the six chromosomes. We use males from suppressor strains of genotype *let-60(n1046)/ let-60(n1046); him-5/him-5; suppressor/suppressor*, to mate with mapping strains of genotype *let-60(n1046)/ let-60(n1046); marker/marker*. The suppressor strain is non-Multivulva, while the marker strains have a marker mutation on the chromosome being tested and are Multivulva and has the marker phenotype (Dumpy or Uncoordinated, for dpy or unc mutations). After the mating, the F1 non-Multivulva, non Marker phenotype animals were selected and they have genotypes of *let-60(n1046)/ let-60(n1046); marker/+; suppressor/+*. We then pick individual F2 non-multivulva animals to individual plates. For a typical mapping experiments, 20 to 40 such animals are selected for each marker used. In the next generation, we first to confirm that the animal picked is indeed suppressed (most or all F3 progeny are nonMultivulva, *let-60(n1046)/ let-60(n1046)*; suppressor/suppressor), then determine if each plate has animals with a marker phenotype (marker/marker). If the suppressor mutation is located on the same chromosome as the marker (and not far from the marker mutation), we do not expect to see animals of the Marker phenotype in any suppressed plates. On the other hand, if the suppressor mutation is located on a different chromosome as the marker gene, the two mutations should segregated independently and we should see animals with a marker phenotype is about 2/3 of the plates. If the suppressor mutation is located on the same chromosome as the marker gene but they are not near each other, we likely to see some plates contain animals with the marker phenotype. Of the 11 suppressors mapped, three were mapped on chromosome I and the rest were mapped on chromosomal IV.

Using three point mapping, which is similar to two point mapping except that the mapping strain contains two mapping markers instead of just one, we were able to define more precise locations within chromosomes for the 11 suppressors. Finally we performed complementation tests to test whether the suppressors were mutations in known genes with similar map positions. Using these methods, we determined that the three suppressors mapping to chromosome I were alleles of the *mek-2* gene and three suppressors mapping to chromosome IV were alleles of *lin-45 raf*. In addition, we showed that four other alleles mapped on the left side of IV and their gene identity has not been determined.

Microinjection transformation:

The chromosome region containing the sur-6 and sur-8 genes is covered by mapped cosmids constructed by the C. elegans genome project. To identify specific cosmid DNA containing the sur-6 or sur-8 gene. We injected cosmids in the region to see which one can rescue the mutant phenotype (methods see above). The host strain has a genotype of *sur-6(ku123) or sur-8(ku167) let-60(n1046); unc-119 (ed3)*. Cosmids were

tested at 5ug/ml together with a transformation marker plasmid consisting of unc-119 genomic DNA insert. Animal transgenic for the marker plasmid will show a non-Unc phenotype and these animals often contain the coinjected cosmid, so can be assayed for rescue. After the injection of gravid adults, F1 progeny with non-Unc were selected. Next generation, stable non-Uncs were selected and scored for percentage of Multivulva phenotype. *let-60(n1046)/ let-60(n1046)* animals are 88% Multivulva and the host strain *sur-6(ku123) or sur-8(ku167) let-60(n1046)/ sur-8(ku167) let-60(n1046)* is less than 5% Multivulva. If the cosmid contains the gene, loss of the gene function by the mutation will then be rescued and result in loss of the suppression (become Multivulva). Methods used for construction double mutant strains are standard methods and will not be described here.

CONCLUSIONS:

Ras-mediated signal transduction pathways play important roles in controlling cell proliferation, differentiation and migration. Mutations in Ras pathway components are associated with the progression of many different types of cancers, including breast cancer. Understanding the regulation of Ras-mediated signaling will lead to a greater understanding of progression and treatment of cancers resulting from pathway deregulation. We describe here the genetic and molecular characterization of two genes, *sur-6* and *sur-8*, which both positively regulate *ras*-mediated signaling during vulval cell fate specification in *C. elegans*.

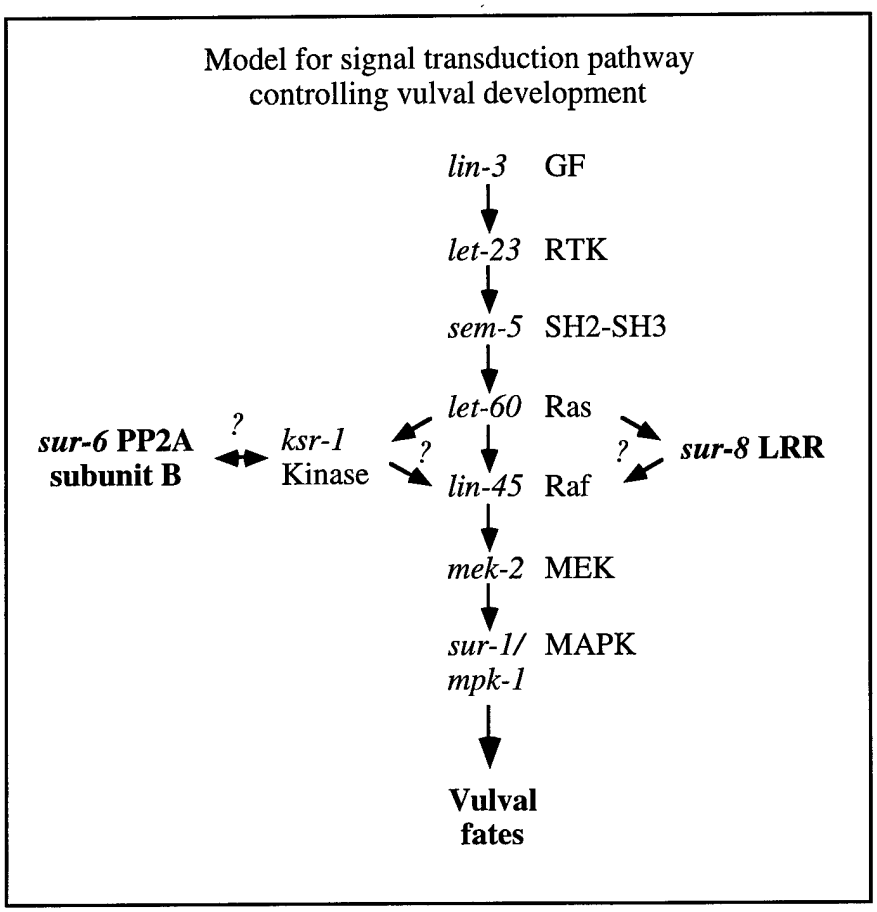
sur-6 (*su*ppressor of *r*as) is defined by a single dominant loss-of-function allele, *ku123*, identified as a suppressor of the Multivulva phenotype caused by an activated *let-60 ras* mutation, *let-60(n1046)*. Genetic studies indicate that *sur-6* is a non-essential, positively acting regulator of *ras*-mediated signaling and it acts in a dosage-dependent manner. Epistasis analysis indicates that *sur-6* acts downstream of or in parallel to *let-60 ras* and upstream of *lin-45 raf*. *sur-6(ku123)* enhances the Vulvaless and Lethal phenotypes caused by loss-of-function mutations in *lin-45 raf* and *sur-1/mpk-1* MAP kinase, but does not synergyse with a strong loss-of-function mutation in *ksr-1* kinase. We propose that *sur-6* acts together with the *ksr-1* at a branch point at the level of *ras* or *raf*. We have cloned *sur-6* by positional cloning and show that it encodes a highly conserved regulatory B subunit of the serine/threonine protein phosphatase 2A.

sur-8 is defined by a single loss-of-function allele, *ku167*, isolated as a suppressor of the Multivulva phenotype caused by an activated *let-60 ras* transgene. Genetic characterization of *sur-8* indicates that, like *sur-6*, it plays a non-essential but activating role in *ras* signaling downstream of or in parallel to *let-60 ras* and upstream of *lin-45 raf*.

However, unlike *sur-6*, *sur-8(ku167)* shows strong synergy with both *ksr-1* and *sur-1/mpk-1* MAP kinase alleles, suggesting that *sur-8* acts in a branch of the pathway distinct from *sur-6* to regulate *ras* signaling activity. *sur-8* has been cloned and is predicted to encode a novel 559 amino acid protein containing 18 leucine rich repeats (LRR) most similar to those found in yeast adenylate cyclase. *sur-8* interacts directly and specifically with *let-60 ras* in the yeast two hybrid system. We have cloned the human homologue of *sur-8* from a human brain library and are currently determining if it shares functional and well as structural homology with *sur-8*.

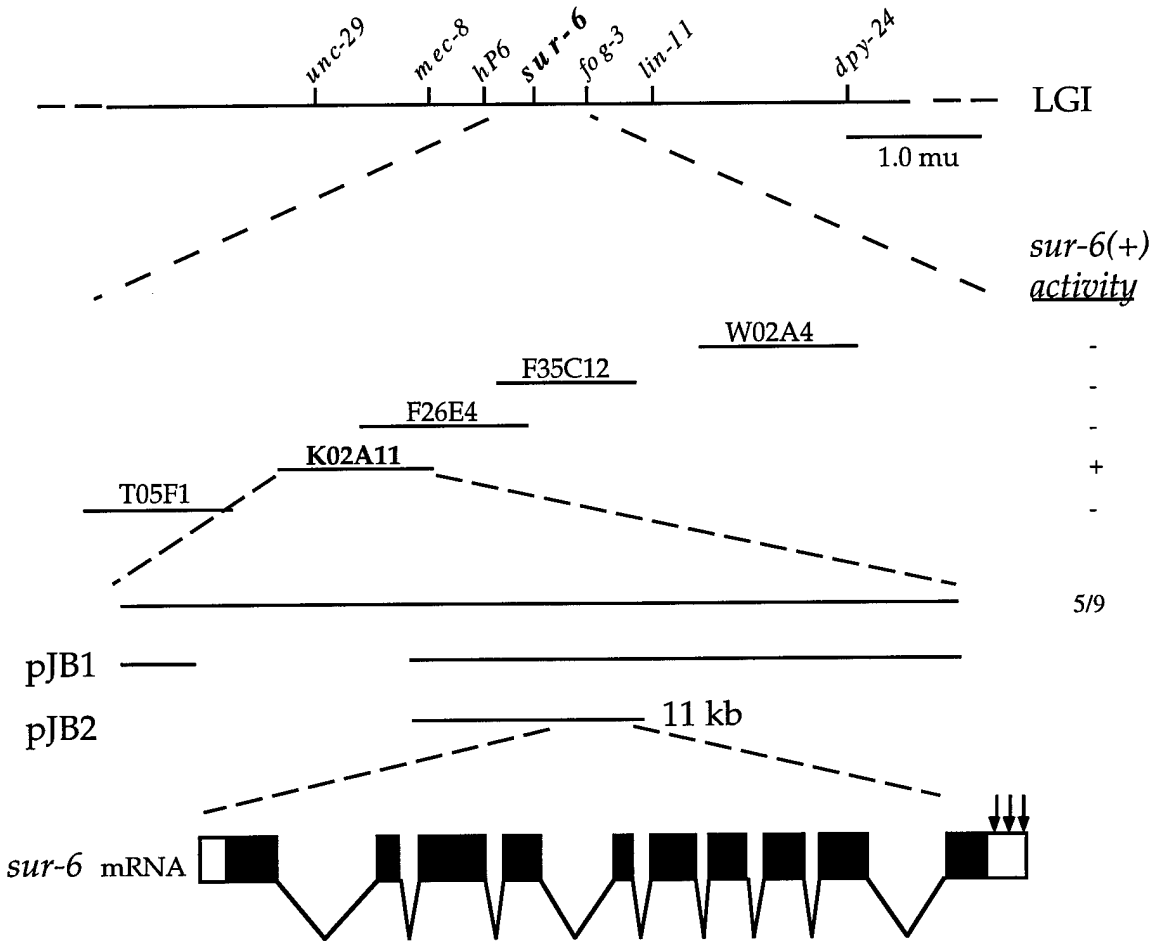
These results provide evidence that *sur-6* and *sur-8* define branch points in the ras pathway feeding into or out of the pathway at the level of ras or raf. The lack of synergy between *sur-6* and *ksr-1* raises the exciting possibility that they may act together, perhaps one acting as a substrate for the other, to positively influence ras signaling. The observation that *sur-8* binds to *let-60 ras* raises the exciting possibility that *sur-8* defines a

second *ras* effector in addition to *lin-45 raf* (see figure below). Current research efforts focus on further characterizing the molecular mechanisms by which *sur-6* and *sur-8* positively regulate ras signaling in hopes of understanding regulation of *ras* signaling in mammalian systems.



APPENDIX:

cloning sur-6:



cloning sur-8: